Oligogalacturonide signalling in plant innate immunity

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ACADEMIC DISSERTATION

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For Love

"He is not mad
His thought is clearer than
The saner man"

-Isis, *Dulcinea*

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ABBREVIATIONS

LIST OF ORIGINAL PUBLICATIONS

This dissertation is based on the following publications, referred to by their Roman numerals in the text. The publications have been reprinted with the kind permission of the respective copyright holders.

I. **Davidsson P***, Broberg M*, Kariola T, Sipari N, Pirhonen M, Palva ET. Short oligogalacturonides induce pathogen resistance-associated gene expression in *Arabidopsis thaliana*. BMC Plant Biol. 2017; 17: 19.

II. Survila M*, **Davidsson P***, Pennanen V, Kariola T, Broberg M, Sipari N, Heino P, Palva ET. 2016 Peroxidase-dependent apoplastic ROS mediates cuticle alterations and functions in DAMP-elicited defense Front Plant Sci. 2016; 7: 1945.

* Equal contribution.

Author's contributions:

I) PD, MB, TK and TP planned and designed the study. PD, MB and NS carried out the experiments. PD and MB analysed the data. PD, MB and TK wrote the paper.

II) P.D, MS, VP, TK and TP planned and designed the study. PD, MS, VP and NS performed the experiments. PD, MS, VP, PH, TK, TP and MB analysed the data. MS was the primary person responsible for writing, figure editing and publication. PD wrote the content related to: Mutant screen, Production of Oligogalacturonides, Growth Inhibition Assay, RNA Extraction and Quantitative RT-PCR Analysis, RNA Sequencing and Detection of ROS. PH and TK reviewed the writing.

ABBREVIATIONS

ABA	abscisic acid
BL	brassinolides
BR	brassinosteroid
CDPK	calcium-dependent protein kinase
CIII Prx	class III peroxidase
CK	cytokinin
DAMP	damage associated molecular patterns
DP	degree of polymerisation
DPI	diphenylene iodonium
ET	ethylene
ETI	effector-triggered immunity
GA	gibberellin
GSEA	gene set enrichment analysis
HR	hypersensitive response
JA	jasmonic acid
MAMP	microbial-associated molecular pattern
MAPK	mitogen-activated protein kinase
MeSA	methyl salicylate
NB-LRR	nucleotide-binding site leucine-rich repeat
NO	nitric oxide
OG	oligogalacturonide
PAMP	microbial-associated molecular pattern
PCWDE	plant cell wall-degrading enzyme
PG	polygalacturonase
PGIP	polygalacturonase inhibiting protein
PGN	peptidoglycan
PR	pathogenesis-related
PRR	pathogen recognition receptor
PTI	pattern-triggered immunity
R	resistance
RBOH	respiratory burst oxidase homologs
RLCK	receptor-like cytoplasmic kinase
ROS	reactive oxygen species
SA	salicylic acid
SAR	systemic acquired resistance
TB	toloudine blue
WAK	wall-associated kinase

ABSTRACT

There are many challenges facing modern agriculture, including but not limited to; climate change, growing population, unsustainable agricultural practises, and the use of potentially harmful insecticides and pesticides. Understanding the plant innate immunity will be essential to developing future sustainable agricultural practises.

Necrotrophic phytopathogens, such as soft rot bacteria, cause large losses in agriculture. Unlike biotrophic pathogens, these typically rely on toxins and plant cell wall-degrading enzymes (PCWDEs) to kill and degrade the host tissue. As such, the methods utilised by the plants to defend themselves against biotrophs, such as the hypersensitivity response (HR), could instead be beneficial for necrotrophic pathogens. One key component in plant defence against necrotrophic pathogens is the recognition of oligogalacturonides (OGs), a breakdown product of the pectin in the plant cell wall, formed by the action of PCWDEs. Similar to direct recognition of the pathogen itself, recognition of OGs trigger a wide array of defence responses, resulting in improved protection against pathogens.

Long OGs with a degree of polymerisation (DP) between 10 and 20 have been well studied. In this study, we explored the role of the relatively less understood short OGs (DP < 9). We utilised trimeric OGs to study the changes induced by short OGs on the transcriptome of *Arabidopsis thaliana*. We established that, similarly to long OGs, short OGs up-regulate genes related to defence and down-regulate genes related to plant growth and development. Phenotypic assays confirmed that pre-treatment with short OGs could improve resistance in *A. thaliana* against the soft rot bacteria *Pectobacterium carotovorum*, to the same degree as long OGs. Furthermore, we showed that treatment with both types of OGs results in seedling growth retardation. As part of investigating the signalling triggered by short OGs, we confirmed that trimeric OGs do not trigger the characteristic initial ROS (reactive oxygen species) burst, but do trigger expression of a large set of peroxidases. Similar to long OGs, part of the signalling in response to short OGs goes via phosphorylation of Mitogen-activated protein kinases (MAPKs). Our results show that short OGs are indeed biologically active elicitors of plant defence, with a signalling pathway that appears to be in part distinct from long OG signalling.

We used the established trade-off between plant defence and plant growth and development to develop screens for mutants with altered OG sensitivity. One mutant line exhibiting hypersensitivity to OGs, resistance to the necrotrophic pathogens *Botrytis cinerea* and *P. carotovorum*, as well as sensitivity to the hemibiotrophic pathogen *Pseudomonas syringae*, was chosen for further studies. We established that the observed phenotypes were due to overexpression a cell wall-localised apoplastic peroxidase (class III peroxidase, CIII Prx) – PEROXIDASE 57 (PER57). We detected increased levels of ROS and increased cuticle permeability, associated with downregulation of genes involved in cutin formation and biosynthesis. We also observed a priming of OG related response genes. The phenotypes could be recaptured by overexpression of several CIII Prxs, indicating a general phenomenon. ABA treatment of these lines restored the phenotypes to wild-type. This appears to be mediated via removal of ROS. Noticeably, the peroxidase activity remained

high in the peroxidase overexpression lines, indicating that while exogenous application of ABA was able to remove the ROS produced by the peroxidases it only had a minor direct effect on the activity of the peroxidases.

Our results, combined with previous research on cuticular and ABA mutants, led us to propose that cuticle integrity is influenced by a positive feed-back loop. A disturbed cuticle leads to elevated ROS levels via increased peroxidase activity, which in turn impairs cuticle formation and biosynthesis. Under normal circumstances this loop is regulated by ABA. In the situation where a necrotrophic pathogen is invading the plant recognition of cell-wall derived DAMPs, such as OGs, it leads to activation of peroxidases that further promote resistance signalling via the creation of ROS.

1 INTRODUCTION

Already in the eighteenth century the influential scholar Thomas Robert Malthus (Malthus, 1798) envisioned a scenario where population increase, following a geometric growth pattern, would outdistance food supply, following an arithmetic growth pattern. In the present day, however, we find ourselves in a situation where we have decreasing numbers of undernourished people in the developing world and food shortages being caused primarily by poor infrastructure and social upheaval (Rosen et al., 2016). The increase in food security is suggested to depend on increased incomes, as well as lowered food prices. This is made possible partially due to the adaptation of modern agricultural practises combined with breeding techniques (Huang et al., 2002).

The intense modern farming currently utilised, however, might be unsustainable in the long term due to degradation of soil resources and environment (Rosset and Altieri, 1997). Modern farming has led to increased consumption of energy, water and fertilizer, as well as increased pollution and losses in biodiversity (Foley et al., 2005). Modern agricultural crops have predominantly been bred for increased yield, possibly at the expense of disease resistance (Lindig-Cisneros et al., 2002; Rosenthal and Dirzo, 1997), and even though modern farming is accompanied by an intense use of insecticides and pesticides, there are vast quantities of crops lost due to insects and pathogens both pre-and post-harvest (Oerke, 2006). The high usage of these chemicals can be potentially harmful to producers and consumers (Antle and Pingali, 1994; Eddleston et al., 2002), as well as have negative effects on biodiversity (Geiger et al., 2010).

Pests and pathogens are predicted to change with global warming, possibly leading to increased pressure on agriculture in northern climates (Bebber et al., 2013). Additionally, human activities are causing global redistribution and the spread of species to new areas (Bebber et al., 2014).

According to the UN DESA report “World Population Prospects: The 2015 Revision” (http://www.un.org) global population is predicted to continue to increase, resulting in increased demands on our ability to sustainably grow food. To be able to achieve that goal, it is essential to understand how plants are able to defend themselves from invaders. Indeed, most plants are resistant to most pathogens they are exposed to in nature and understanding how the plants are able to achieve this remarkable phenomenon will be essential in creating sustainable agricultural practises (Dangl and Jones, 2001; Heath, 2000).

1.1 Phytobacteria

Plant pathogens are typically classified based on their method of acquiring nutrients from the plants. Unlike biotrophs, who rely on leeching nutrients from the living cells, necrotrophs kill the plant cells in order to acquire nutrients (Collmer et al., 2009; Mengiste, 2012).

Hemibiotrophs rely on an initial biotrophic phase, followed by a shift during later stages to a necrotrophic mode.

Biotrophs, and hemibiotrophs such as *Pseudomonas syringae*, initially rely on a stealthy approach, where the plant cells are kept alive while the pathogen avoids or supresses the plant immune system using effector proteins (Collmer et al., 2009; Göhre and Robatzek, 2008; Kay and Bonas, 2009; Niks and Marcel, 2009). As these effectors often supress very specific responses, possibly limited to particular host species (Spanu et al., 2010), (hemi)biotrophic pathogens tend to be relatively host specific.

Necrotrophs typically utilise toxins and a wide array of plant cell wall-degrading enzymes (PCWDEs) to degrade the plant tissue and, as a result of their infection tactic, typically have a wide host range (Davidsson et al., 2013). Bacterial soft rot are agriculturally important diseases causing significant losses both pre- and post-harvest (Davidsson et al., 2013) Even though soft rot bacteria, such as such as the *Dickeya* and *Pectobacterium* genera, are traditionally seen as necrotrophs it seems that the necrotrophic stage is preceded by a stage more reminiscent of the biotrophic lifestyle (Liu et al., 2008; Toth and Birch, 2005). Relatively little is known about this initial phase. *Pectobacterium carotovorum* has one of the widest host ranges of all soft rot bacteria and cause large losses, especially in the economically important potato crop (Toth et al., 2003). To date no effector that functions by suppressing the immune system has been identified in *P. carotovorum*. The effectors analysed so far, e.g. DspE, promotes cell death, disease progression and plant tissue maceration (Kim et al., 2011). The difference in strategy between biotrophs and hemibiotrophs highlights the complexity in the responses required by the plant innate immunity.

1.2 Plant innate immune system

The foremost preformed defences of plants are various structural and physical barriers, such as the cell wall and cuticle (Freeman, 2008), but can also include antimicrobial compounds such as glucosinolates (Wittstock and Gershenzon, 2002). The preformed defences are not rigid and unchanging, but instead can be influenced and strengthened by activation of the plant innate immune system. The plant innate immune system shares many features with the innate immune system in animals (Ausubel, 2005), including receptors for microbe-associated molecules, mitogen-associated kinase signalling cascades and production of antimicrobial peptides.

One of the most influential conceptual models of plant innate immunity in recent years is the well-known “zigzag”-model (Jones and Dangl, 2006). Briefly, the model envisions two separate branches of the immune system. The first branch is characterised by an early recognition of evolutionary conserved microbial/pathogen-associated molecular patterns (MAMPs/PAMPs) by pathogen recognition receptors (PRRs), resulting in pattern-triggered immunity (PTI). PTI can also be triggered by endogenous damage associated molecular patterns (DAMPs) released during infection (Boller and Felix, 2009). Successful pathogens

have evolved effectors that can interfere with PTI. The second branch is characterised by the recognition of these effectors by specific disease resistance (R) genes (typically nucleotide-binding leucine-rich repeat (NB-LRR) proteins), resulting in effector-triggered immunity (ETI). Again, successful pathogens have evolved by modifying these effectors or by producing new effectors targeting ETI. Generally, ETI is considered to be a stronger response, typically triggering the hypersensitive response (HR), and to be pathosystem-specific.

Several recent publications have discussed the limitations of the zigzag-model (Cook et al., 2015; Pritchard and Birch, 2014, etc.). Some of these limitations are:

- In reality there is no clear separation between MAMPs and effectors. Effectors can be widespread, just like MAMPs and contain conserved patterns functioning as MAMPS. One such example is the necrosis and ethylene-inducing peptide 1 (Nep1) and its homologs Nep1-like proteins (NLPs) (Böhm et al., 2014; Oome et al., 2014). Many NLPs in different phylogenetic kingdoms share an immunogenic nlp20 peptide motif. Since effectors may elicit defence responses and MAMPs may be required for virulence, single components does not necessarily belong to a specific group (Thomma et al., 2011).
- Strong PTI responses resulting in HR is observed in many cases, with for example the classical MAMP flg22 epitope of flagellin causing HR in the model plant, *Arabidopsis thaliana* being the most well-known (Naito et al., 2007). Vice versa, weak ETI responses are abundant, with recognition of the *P. syringae* type III effector AvrRps4 by RPS4 as an example (Thomma et al., 2011). As such there is no clear difference in the strength of the responses of PTI and ETI.
- Not only NB-LRR proteins, but also PRRs seem to be under continual evolutionary pressure and MAMPs are not as stable as the model would indicate. One such evolutionary MAMP-PRR pair is the well-studied FLAGELLIN-SENSITIVE 2 (FLS2) receptor, recognising flg22 (Gómez-Gómez and Boller, 2000). Variation in the FLS2 receptor has been observed in several instances. For example, the *A. thaliana* WS-0 ecotype does not contain a functional FLS2 allele and consequently does not respond to flg22 (Bauer et al., 2001; Zipfel et al., 2004). Furthermore, plants can use other receptors to recognise different epitopes of flagella (Cai et al., 2011) Differences in the sequence of the flg22 epitope has been observed (Clarke et al., 2013), as well as posttranslational modification of flagellin (Taguchi et al., 2006), affecting the flg22-FLS2 interaction. The same situation also seems to be the case for ELONGATION FACTOR THERMO UNSTABLE (EF-TU), with the well-studied epitope elf18, and its receptor EF-TU RECEPTOR (EFR) (Furukawa et al., 2014; Kunze et al., 2004), highlighting that this is most likely a general phenomenon.
- Not all NB-LRR proteins are pathosystem-specific. For example, Rxo1 in maize can confer resistance to several unrelated bacteria (Zhao et al., 2004a, 2004b) and Mi-1.2 in tomato can confer resistance to insects as well as nematodes (Rossi et al., 1998; Vos et al., 1998), indicating that NB-LRRs can also be evolutionary conserved.
- The model is of limited use in visualising symbiotic and necrotrophic interactions.

Given that this model is an important conceptual tool, not least for visualising the evolutionary relationship between plants and pathogens, it is also essential not to oversimplify. Real interactions depend on the specific trigger(s), receptor(s), environmental conditions, as well as the status and history of the plant – not on whether the trigger is classified as a MAMP, DAMP, elicitor or toxin.

1.3 Microbial associated molecular patterns

Recognition of MAMPs typically trigger responses such as; production of reactive oxygen species (ROS) and reactive nitrogen species, cell wall modification and induction of pathogenesis-related (PR) proteins, as well as antimicrobial compounds (Newman et al., 2013). The study of MAMPs and their corresponding receptor complexes have received a high degree of focus during the last few years. Recognition of an epitope by the corresponding receptor typically leads to highly complex phosphorylation events, of which the details are still being unravelled (Macho and Zipfel, 2014). One well-studied model MAMP is the 22 amino acid epitope flg22, constituting a conserved part of bacterial flagella (Felix et al., 1999), for which FLS2 was identified as the receptor in Arabidopsis (Gómez-Gómez and Boller, 2000). Recognition of flg22 leads to complex formation with BRI1-ASSOCIATED RECEPTOR KINASE 1 / SOMATIC EMBRYOGENESIS RECEPTOR KINASE 3 (BAK1/SERK3), functioning as a co-receptor essential for flagellin signalling (Chinchilla et al., 2006, 2007). This dependence on BAK1, or other SERK proteins, as co-receptors appear to be a common phenomenon observed for example also for the MAMP elf18 and its receptor EFR (Roux et al., 2011), as well as for recognition of the phytohormone brassinosteroid (BR) by BRASSINOSTEROID INSENSITIVE 1 (BRI1) (Nam and Li, 2002). In the absence of flg22, BAK1-INTERACTING RECEPTOR-LIKE KINASE 2 (BIR2) interacts with BAK1, preventing the association with FLS2 (Halter et al., 2014). Interestingly, the recognition of chitin by CHITIN ELICITOR RECEPTOR KINASE (CERK1) causes homo-dimerization and does not require the recruitment of BAK1 (Greeff et al., 2012). However, LYSM-CONTAINING RECEPTOR-LIKE KINASE 5 (LYK5) appears to be essential for chitin binding and complex formation (Cao et al., 2014).

Further, several Receptor-like cytoplasmic kinases (RLCKs) appear to play a major role as positive regulators, with different PRRs recruiting a different set of RLCKs and several of them interacting with more than one PRR complex (Macho and Zipfel, 2014). The most well-known of these is probably BOTRYTIS-INDUCED KINASE1 (BIK1), which, in addition to phosphorylating the various components in the receptor complexes, also participates in activating the NADPH oxidase RESPIRATORY BURST OXIDASE HOMOLOG D (RBOHD). Similarly, there appears to be protein phosphatases interacting with the PRR complexes acting as negative regulators, with the most well know example being KINASE-ASSOCIATED PROTEIN PHOSPHATASE (KAPP) (Ding et al., 2007).

It is now well understood how PRRs are able to sense complex structures present in the pathogens, one suggestion is that these structures are continuously being built up and broken

down as part of the pathogen life cycle. Another possible scenario is that plants are able to produce enzymes to break down the insoluble structures into soluble PRR ligands. Arabidopsis produces a hydrolase, LYSOZYME 1 (LYS1), capable of releasing soluble peptidoglycan (PGN) fragments from insoluble bacterial cell walls (Liu et al., 2014). In Arabidopsis, PGN fragments have been shown to be sensed by a receptor complex consisting of Lysin motif (LysM) domain proteins LYM1 and LYM3 and CERK1 (Willmann et al., 2011). Most likely LYM1 and LYM3 act in ligand recognition and binding and CERK1 mediates transmembrane signal transduction.

1.3.1 Damage associated molecular patterns

Besides being able to recognise motifs belonging to various microbes, both plants and animals are able to sense endogenous molecular patterns that are released during infection, or by tissue damage from insect or herbivores (Boller and Felix, 2009). Such damage associated molecular patterns (DAMPs) trigger responses similar to those triggered by MAMPs (Boller and Felix, 2009), and exist in several forms. A simplified model of MAMP/DAMP recognition upon infection with *Pectobacterium* is visualised in Figure 1.

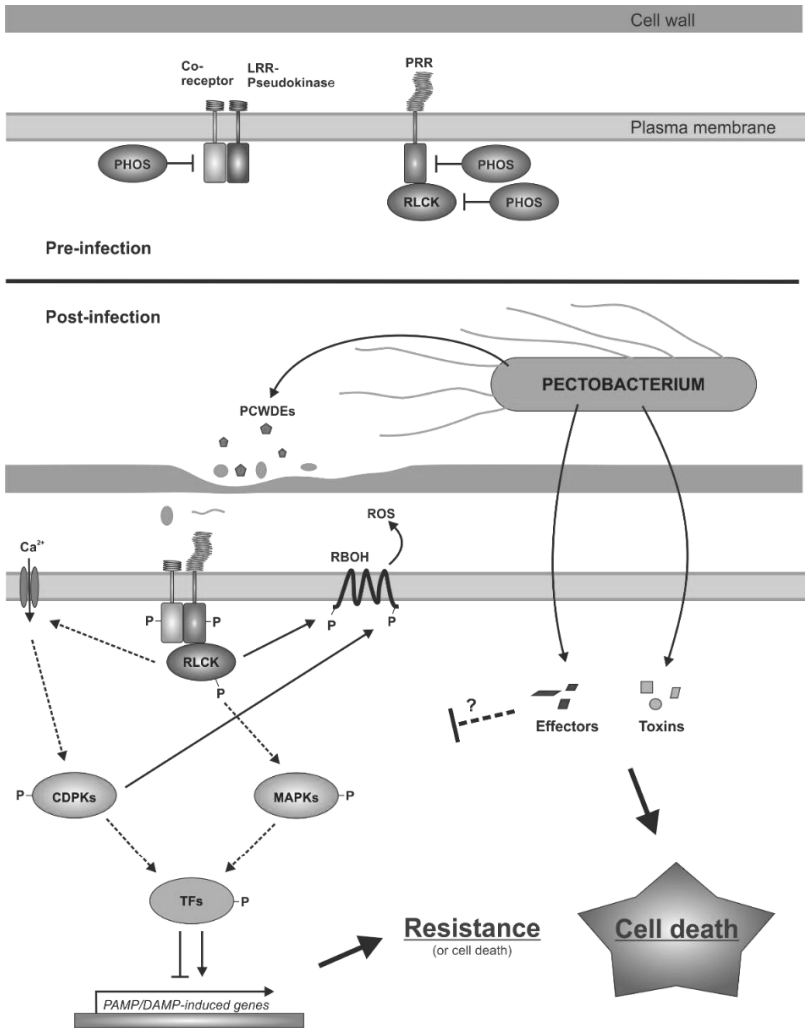

Figure 1: General model of MAMP/DAMP recognition upon infection with Pectobacterium based on FLS2 in Arabidopsis. In the unchallenged state PRRs, Co-receptors and RLCKs are kept under negative regulation by various Phosphatases (PHOS). Association between Co-receptors and PRRs can be inhibited by binding to LRR-Pseudokinases. Pectobacterium can be sensed via a wide array of components, in this case illustrated by flagella and break-down products of the cell wall release by the action of PCWDEs. Recognition of the specific receptor ligand by a PRR results in hetero-dimerization with potential Co-receptors. This in turn triggers various phosphorylation events, leading to Ca^{2+}-influx, ROS production and activation of various defence-related genes. Typically, this is associated with resistance to the pathogen. However, under certain circumstances the system could also lead to cell death and proceeding pathogen invasion. Besides PCWDEs the pathogen delivers toxins and effectors targeting the plant. The effectors could either function by promoting cell death or by inhibiting various components in the defence signalling. Modified from Couto and Zipfel, 2016 and Davidsson et al., 2013.

1.3.2 Peptide-based DAMPs

One of the most well studied categories of DAMPs is shorter peptides produced from larger precursor proteins upon damage or infection. They were first discovered as systemin in tomato, but has since been identified in many Solanaceous species (Hind et al., 2010; Pearce et al., 1991).

Among the peptide-based DAMPs, the plant elicitor peptide (Pep) family consisting of eight members (Yamaguchi and Huffaker, 2011). Pep1 and Pep2 are recognised by PEP RECEPTOR 1 (PEPR1) and PEPR2 (Yamaguchi et al., 2006, 2010). It is still unclear whether the Peps have redundant or specialised function and the enzymes that release active peptides from their precursors have not been identified. PEPR1 and PEPR2 function with the co-receptors BAK1/SERK3 and BAK1-LIKE1 (BKK1)/SERK4 (Roux et al., 2011; Yamaguchi and Huffaker, 2011). These peptides appear to play an important role in signalling by other MAMPs and DAMPs (Gravino et al., 2016; Yamada et al., 2016).

1.3.3 Extracellular ATP and re-located proteins

Certain compounds that have a regular function when the plant is not stressed or under attack can act as DAMPs when they are present in the wrong cellular compartment, possibly caused by cell damage or cells undergoing HR. One such example in Arabidopsis is the HIGH MOBILITY GROUP BOX 3 (HMBG3) protein (Choi and Klessig, 2016). HMBG3 appears to function as a typical DAMP when released into the apoplast. Interestingly, this function seems to be inhibited by binding to salicylic acid (SA) and as such could be a player in the SA - jasmonic acid (JA) interaction during plant defence against pathogens.

The protein DOES NOT RESPOND TO NUCLEOTIDES 1 (DORN1) was recently found to be a receptor for extracellular ATP (Choi et al., 2014). DORN1 has been proposed to play a role in wounding, but it is not yet clear if it plays a role in plant-pathogen interactions (Tanaka et al., 2014).

1.3.4 Oligogalacturonides

Oligogalacturonides (OGs) constitute one of the most well studied types of DAMPs. They are biologically active carbohydrates (oligosaccharins) resulting from the breakdown of homogalacturonan, a major component of pectin (Côté and Hahn, 1994; Ridley et al., 2001). OGs have been seen to trigger typical PTI responses, including general responses such as; oxidative burst, fortification of the cell wall, production of phytoalexins and proteinase inhibitors, as well as hormone biosynthesis (Ridley et al., 2001). Like many other DAMPs, OGs trigger PTI in both monocots and dicots, and could be said to be part of an evolutionary old system for sensing danger (Baker et al., 1990; Côté and Hahn, 1994; Randoux et al., 2009, 2010).

Pathogen-generated polygalacturonases (PGs) play an important role in the sensing of an ongoing infection, either by being directly perceived by plant receptors (Zhang et al., 2014), or through sensing of the resulting OGs generated by their enzymatic action (Brutus et al., 2010). These OGs are present during infection in a varied degree of polymerization (DP), typically ranging from 2 to over 20 monomers linked together (Bartling et al., 1995; Forrest and Lyon, 1990; Pontiggia et al., 2015; Preston et al., 1992; Roy et al., 1999). The PGs generally target non-methylated polygalacturonan. In *P. carotovorum* the endo-polygalacturonase PehA is one of the major players carrying out this function, whereas *Dickeya dadantii* only has exo cleaving polygalacturonases (Hugouvieux-Cotte-Pattat et al., 2001; Kotoujansky, 1987; Saarilahti et al., 1990). Furthermore, PCWDs such as PGs, are not only produced by necrotrophic pathogens but also play a critical role during the colonisation of plant roots by symbiotic rhizobia and OGs appear to play a role in the initial Rhizobium-Legume interactions (Moscatiello et al., 2012).

Part of the OG induced responses is the induction of PG inhibiting proteins (PGIPs). As the name implies, these PGIPs directly inhibit the activity of PGs. It has also been suggested that this activity increases the quantity of higher DP oligogalacturonides thought to be more biologically active in defence against the fungal necrotroph *Botrytis cinerea* (De Lorenzo et al., 2001; Decreux and Messiaen, 2005). The role of PGIPs in the defence against bacterial pathogens has not been as well studied, but has been seen to be important in resistance of Chinese cabbage against *P. carotovorum* (Hwang et al., 2010). Further, PGIPs from tomato have been shown to be capable of inhibiting PGs from *Ralstonia solanacearum* (Schacht et al., 2011).

1.3.5 Oligogalacturonide perception

Although OGs were the first oligosaccharins characterised (Bishop et al., 1981; Hahn et al., 1981), the study of OG signalling has historically been difficult due to the complexity of OG responses (Ridley et al., 2001). The Wall-associated kinases (WAKs) have long been seen as possible candidates as OG receptors. However, silencing of the WAK gene family results in lethality, probably due to their involvement in regulation of growth and development (Wagner and Kohorn, 2001). Furthermore, a high redundancy and tight genetic linkage between the different WAKs has complicated the study of these potential receptors (Brutus et al., 2010; Gramegna et al., 2016). The importance of WAKs in OG signalling is supported by several lines of inquiry. First, both WAK1 and WAK2 have been shown to bind to pectin in vitro (Kohorn et al., 2009) and WAK1 binds specifically to OGs (Cabrera et al., 2008; Decreux and Messiaen, 2005; Morris et al., 1982). The in vitro binding of OGs to WAK1 was seen to require a DP over nine, and more particularly, the binding required formation of a calcium-induced conformation known as an egg box dimer. The egg box form progressively, and there seems to be two different forms of perception systems in which WAK1 can bind these dimers (Cabrera et al., 2008). Also, OGs with a lower DP can form egg box dimers. However, when formed by shorter chain OGs, these are unstable and easily disrupted by competing monovalent ions. Secondly, gene expression studies indicate that

WAK1 is up-regulated by wounding and exogenous application of OGs (Denoux et al., 2008; Wagner and Kohorn, 2001). The essential proof that WAK1 was capable of acting as an OG receptor came from utilising a domain swap approach (Brutus et al., 2010), in which chimeric receptors of EFR and WAK1 was used to show that the WAK1 ectodomain could be triggered by long chain OGs to activate the EFR kinase domain, and vice versa that the EFR ectodomain could be triggered by the elf18 peptide to activate the WAK1 kinase domain, resulting in a defence response mimicking a normal OG response. In line with the proposed role of WAK1 as an OG receptor, plants overexpressing this protein were more resistant to *B. cinerea*.

WAK1 has been shown to form a complex with KAPP and GLYCEINE-RICH PROTEIN 3 (GRP-3) (Anderson et al., 2001; Park et al., 2001). The biological relevance of these interactions have been demonstrated with individual loss of function mutants of KAPP and GRP3 leading to prolonged OG responses and resistance to *B. cinerea* (Gramegna et al., 2016). Overexpression of KAPP confirms that this protein functions as a negative regulator of defence responses. However, overexpression of GRP-3 indicates that this protein could negatively regulate flg22 responses and enhance OG responses. Intriguingly, loss of GRP-3 or WAK1 overexpression did not affect resistance against *P. carotovorum*, whereas loss of KAPP lead to increased sensitivity. This indicates that there are differences in OG signalling in response to *P. carotovorum* and *B. cinerea*, possibly reflecting the needs of the plants to be able to differentiate between different types of necrotrophic pathogens.

It has been reported that activation of WAK1 and several biological responses appear to be dependent on OGs with a DP between 10 and 15 (Brutus et al., 2010). However, several studies indicate that OGs with a lower DP might also trigger plant responses such as; induction of genes involved in JA biosynthesis (Norman et al., 1999), induction of ethylene production (O'Donnell et al., 1996; Simpson et al., 1998), production of proteinase inhibitors (Moloshok et al., 1992; O'Donnell et al., 1996; Thain et al., 1990), depolarisation of leaf mesophyll cells (Thain et al., 1990), induction RLCKs (Montesano et al., 2001) and induction of resistance against *P. carotovorum* in potato (Weber et al., 1996; Wegener et al., 1996). Moreover, short OGs have been seen to have a developmental effect in strawberry plants (Miranda et al., 2007). As WAK1 activation might be dependent on longer OGs, the method by which the plants are able to sense short OGs remains to be elucidated.

1.3.6 Oligogalacturonide signalling

Similar to most plant responses, the OG responses appear to be tightly linked to phytohormone regulation. Due to the connection between growth and development on one side, and plant-pathogen interactions on the other, it is not possible to entirely ignore the growth-related role of OGs when studying the role in plant innate immunity. Exogenous application of OGs was early on observed to cause an inhibition of auxin-induced stem elongation (Branca et al., 1988). Since then the antagonistic effect on auxin signalling has been solidified, but the mechanism remains elusive (Falasca et al., 2008; Qi et al., 2012; Savatin et al., 2011). In Arabidopsis, enhanced plant resistance to *B. cinerea* induced by OGs

seems to be independent of SA and JA and dependent on ethylene (ET), PHYTOALEXIN DEFICIENT 3 (PAD3) and the accumulation of the phytoalexin camalexin (Ferrari et al., 2007; Gravino et al., 2015). The ET dependent signalling, in turn, appears to be dependent on Calcium-dependent protein kinases (CDPKs).

Headway into the details of OG signalling has been made with several studies looking at the changes in transcriptome induced in *A. thaliana* treated with exogenously applied long chain OGs, and comparing this response with the response to the MAMP flg22, as well as infection with *B. cinerea* (Ferrari et al. 2007; Moscatiello et al. 2006; Denoux et al. 2008). It should, however, be noted that the studies by Ferrari et al. and Denoux et al. used an OG-mixture enriched in long OGs where the average DP was between 10-15, but it also contained fractions of shorter OGs. Moscatiello et al, however, used purified OGs with a DP between 10 and 15.

The first genome wide transcriptome analysis of OG responses used mesophyll cell suspension cultures and focused on investigating calcium-dependent and independent signalling pathways (Moscatiello et al., 2006). The study showed that OG-induced activation of genes involved in ET signalling required both pathways, whereas activation of JA-responsive genes mainly appeared calcium-dependent, in agreement with an earlier study (Hu X. et al., 2003). It would also seem that protein kinase-dependent phosphorylation is involved in the early stages of OG signalling (Moscatiello et al., 2006). The use of cell cultures could limit the biological value of the data (Sato, 2013), and later studies have utilised Arabidopsis seedlings and whole plants. This is probably why the overlap in gene expression between this study and others is minimal.

A later study (Ferrari et al., 2007) , compared the responses induced by OGs with responses induced by infection with *B. cinerea*. Overall, somewhat similar responses were observed with approximately 50% overlap. They further established that OG-induced resistance to *B. cinerea* was independent of JA, ET and SA signalling and dependent on PAD3. However, the same group later demonstrated that ET signalling was indeed important for OG-induced resistance to *B. cinerea* (Gravino et al., 2015). The transcriptomic study was further expanded by comparing the OG induced gene expression with the induction caused by flg22 treatment (Denoux et al. 2008). Even though the responses were found to be similar, especially at early time points, the response to flg22 was found to be stronger and of a more prolonged nature. Interestingly, both treatments activated multiple components of ET, JA and SA pathways. Several SA-dependent genes were, however, found to be significantly induced by only flg22 and not with long chain OGs. Of these, somewhat surprisingly, PR1 one was found not to be induced by OGs. This is in contrast to an earlier study looking at the Arabidopsis response to mixed length OGs where calcium and hydrogen peroxide-dependent induction of several defence related marker genes; *CHS*, *GST*, *PAL* and noticeably *PR1,* was observed (Hu et al., 2004). Further complicating the issue, a recent study (Gravino et al., 2016) using a seemingly identical experimental set-up as Denoux et al. 2008, saw a clear strong induction of PR1 (approximately 30-fold). This highlights the variability of OG responses, as well as their dependency on the exact experimental set-up. Interestingly, even though the responses to OGs and flg22 are similar, one study indicates that OG (DP6-20) signalling might have a mutually antagonistic relationship with both flg22 and elf18 (Aslam

et al., 2009). This was seen as reduced calcium influx and reduced ROS burst upon concurrent treatment. Curiously, this study did not see any upregulation of PR1 by OGs.

It would be tempting to speculate that OG dependent responses rely more on the JA/ET-dependent signalling than SA-dependent signalling, in agreement with resistance in Arabidopsis to *P. carotovorum* (Doares et al., 1995; Norman et al., 1999; Norman-Setterblad et al., 2000; Palva et al., 1993; Vidal et al., 1997). This argument is further strengthened by studies highlighting jasmonates, and other oxylipins, as having a central role in defence responses following tissue damage. These have been proposed to mediate the induction of defence in response to OG signals generated by pathogen or herbivore attacks (Farmer and Ryan, 1992). This, however, is most likely an oversimplification and the role of SA signalling, as well as other signalling pathways, should not be overlooked (Vidal et al., 1998).

Recent studies looking at protein phosphorylation in response to OGs have found phosphorylation of proteins belonging to various functional classification such as; kinases, phosphatases, RLCKs, heat shock proteins, ROS scavenging enzymes, cellular trafficking, transport, as well as general defence and signalling (Kohorn et al., 2016; Mattei et al., 2016). Similarly to the results from transcriptomic experiments, these studies highlight distinct but overlapping responses between OG and flg22 perception.

Recent headway into elucidating the OG signalling pathway(s) has further set OG signalling apart from that of MAMP signalling, such as signalling triggered by flg22 and elf18 (Gravino et al., 2016). BAK1/SERK3 and the closest paralog BKK1/SERK4 are involved in response to flg22 and other MAMPS such as; HrpZ, peptidoglycan, lipopolysaccharide and Pep1, as well as BR-dependent signalling. However, they do not appear to be involved in NECROSIS-INDUCING *PHYTOPHTHORA* PROTEIN 1 (NPP1) and chitin signalling. AvrPto is a well-studied effector found in *P. syringae* that is capable of counteracting immunity resulting from recognition of flg22 and elf18 by inhibiting the kinase function of FLS2, EFR and co-receptors BAK1 and BKK1 (Xiang et al., 2008). Gravino and co-workers were able to demonstrate that AvrPto is also capable of inhibiting OG signalling and that BAK1 and BKK1 are indeed involved in OG signalling as well. However, only a subset of the analysed OG responses were affected by AvrPto and only a subset of those responses were affected by dual loss of BAK1 and BKK1. Furthermore, they were able to demonstrate that signalling through the DAMPs Peps and their PEPR receptors contribute to the OG responses. This research further highlights the complexity of OG signalling, indicating multiple and partially redundant modes of sensing OGs. Again, whether this is dependent on different length of OGs being sensed by separate recognition complexes remains to be elucidated.

Complicating the issue further; nitric oxide (NO) appears to play a role in OG signalling (Rasul et al., 2012). It was found that OGs trigger NO production in Arabidopsis and this NO production was in turn found to be important for OG-induced immunity to *B. cinerea*. Whether OG-induced NO production plays a role in immunity towards bacterial necrotrophs remains to be investigated.

1.3.7 Cellulose oligomers

It has been proposed that the breakdown products of other cell wall polymers, besides pectin, may act as DAMPs and recent data suggests that short (DP 2-4) cellulose oligomers may also act as DAMPs (Souza et al., 2017). Interestingly, these cellulose fragments do not trigger the characteristic ROS burst, nor do they trigger callose deposition. However, exogenous application triggers qualitatively similar gene expressions to other MAMPs/DAMPs, as well as primes the plant defences against subsequent pathogen infection. At least part of the short cellulose signalling seems to go through MAPK signalling, as indicated by MITOGEN-ACTIVATED KINASE 3 (MPK3) and MPK6 phosphorylation. Interestingly, the responses to short cellulose fragments appear to be synergistic with other MAMPs and DAMPs.

1.4 Hormonal crosstalk in defence signalling

Phytohormones are typically seen as central mediators of plant growth, development and responses to abiotic stress, as well as plant defences. As such, they form the basis of a complex crosstalk playing a key role in determining the outcome of virtually all plant-pathogen interactions (Dong, 1998; Robert-Seilaniantz et al., 2011; Wang and Irving, 2011).

In simplified models JA and ET signalling has been said to promote resistance against necrotrophic pathogens and herbivores, whereas SA has been seen as the hormone responsible for defence signalling resulting in increased resistance against bio- and hemibiotrophic pathogens (Glazebrook, 2005; Robert-Seilaniantz et al., 2011). These two pathways tend to be seen as mutually antagonistic, with SA-signalling inhibiting JA/ET-signalling and vice versa. However, this is obviously an over-simplistic view and several contradictory lines of evidence exist, for example, it was noted over two decades ago that SA could induce resistance against *P. carotovorum* in tobacco (Palva et al., 1994). Also, several other hormones play a role in the plant-pathogen interactions and the outcome is the result of a finely tuned balance and interplay between many actors. Part of the effect of these other hormones on pathogen defence appear to be mediated through interactions with SA and JA/ET pathways. A common theme for the regulation of many hormonal pathways is that they are mediated through ubiquitination and subsequent proteosomal degradation of negative regulators (Robert-Seilaniantz et al., 2011). As a result of the role of phytohormones in plant defence, many pathogens have evolved the ability to manipulate hormonal signalling in plants, either by directly producing hormones or hormone mimics, or by influencing the hormonal crosstalk. (Costacurta and Vanderleyden, 1995; Robert-Seilaniantz et al., 2011). Among soft rot bacteria, *Dickeya dadantii* has been shown to produce auxin (Yang et al., 2007). In the case of *Pectobacterium*, no direct evidence for virulence determinants affecting plant hormones has been reported so far.

1.4.1 Salicylic acid

SA is a phenolic acid that functions as a signal for the activation of both local and systemic acquired resistance (SAR), a well-studied response causing plant-wide enhanced defence in response to infections (Wang and Irving, 2011). In Arabidopsis, SA is sensed through NONEXPRESSER OF PR GENES 1 (NPR1) and its paralogues NPR3 and NPR4 (Attaran and He, 2012; Fu et al., 2012; Kumar, 2014). A possible mobile signal for SAR was identified as methyl salicylate (MeSA) (Park et al., 2007).

Mechanistically NPR1 forms oligomers in the cytoplasm where increased levels of SA disrupt the NPR1 oligomers into monomers. This allows NPR1 to relocate to the nucleus and activate transcription of SA responsive defence genes, such as the PR proteins. NPR1 is phosphorylated upon its interaction with transcription factors and proteasome mediated degradation of phosphorylated NPR1 is important in triggering expression of SA responsive genes.(Kumar, 2014).

ROS signalling is involved both upstream and downstream of SA signalling in response to stress, and activation of SA signalling in stressed plants is preceded by an increase in ROS (Herrera-Vásquez et al., 2015). Since not all ROS production promotes SA signalling, it is unclear how the specificity of ROS signals in triggering SA biosynthesis is established. Moreover, SA is able to bind SALICYLIC ACID BINDING PROTEINS (SABPs) and inhibit their normal function as ROS scavengers, resulting in increased ROS accumulation (Kumar, 2014). SA also appears to be able to increase ROS scavenging, however, and as such is critical in constraining ROS signalling (Herrera-Vásquez et al., 2015). This dual effect on ROS is thought to result in temporal dynamics where at first SA promotes ROS and later inhibits ROS.

1.4.2 Jasmonates

JA and its derivatives are cyclic fatty acid-derived regulators synthesised from linolenic acid and have been seen to play major roles in both growth and development, as well as plant defence (Ludwig-Müller, 2011). Most JA responses are mediated by the JA receptor F-box protein CORONATINE INSENSITIVE 1 (COI1) (Robert-Seilaniantz et al., 2011) and a majority of the JA signalling in responses to pathogens goes through MYC2 and ETHYLENE RESPONSE FACTOR 1 (ERF1) when ET is present (Lorenzo et al., 2003). JASMONATE ZIM-domain (JAZ) transcriptional repressor proteins suppress JA signalling via binding of MYC2 and COI1. Binding of JA-Ile to SCF^{COI1} complexes leads to degradation of JAZs through ubiquitin-mediated proteosomal degradation (Robert-Seilaniantz et al., 2011).

In Arabidopsis, JA signalling is often visualised as consisting of two antagonistic pathways; the MYC branch primarily responding to wounding and herbivorous insects and the ERF branch primarily responding to necrotrophs (Pieterse et al., 2012; Schmiesing et al., 2016). The latter requires activation of the ET signalling pathway. It is worth noting that also the

MYC branch plays a role in plant-pathogen interactions and can function in priming plants for enhanced pathogen defence (Pozo et al., 2008).

1.4.1 Ethylene

ET is a gaseous hormone involved in various processes such as; fruit maturation, germination, senescence and growth. But it has also traditionally been associated with plant defence (Wang and Irving, 2011).

In Arabidopsis, ET is sensed by the histidine kinases ETHYLENE RESPONSE 1 (ETR1), ETR2, ETHYLENE RESPONSE SENSOR 1 (ERS1), ERS2 and ETHYLENE INSENSITIVE 4 (EIN4), located in the endoplasmic reticulum (Robert-Seilaniantz et al., 2011). They function as negative regulators of ET signalling in the absence of ET. Binding of ET results in reduced activity of the associated protein kinase CONSTITUTIVE TRIPLE RESPONSE 1 (CTR1). This results in downstream transcriptional changes leading to ET responses (Gallie, 2015; Lacey and Binder, 2014). The transcription factors EIN2 and EIN3 are stabilised by ET, by promoting proteosomal degradation of F-box proteins, which in the absence of ET targets EIN2 and EIN3 for proteosomal degradation via ubiquitination. EIN3 regulates, among others, ORA59 and ERF1, which are involved in both ET and JA signalling.

1.4.2 Auxin

There are several forms of endogenous auxins. All auxins are compounds with an aromatic ring and a carboxylic acid group (Ludwig-Müller, 2011). Auxin has traditionally been studied for its key role in plant growth and development. Lately the role of auxin in plant-pathogen interactions has been the target of several investigations (Fu and Wang, 2011; Kazan and Manners, 2009; Naseem et al., 2015a, etc.) The F-box protein TRANSPORT-INHIBITOR-RESISTANT1 (TIR1) has been identified in Arabidopsis as a receptor for auxin (Dharmasiri et al., 2005; Kepinski and Leyser, 2005). Also, AUXIN BINDING PROTEIN 1 (ABP1) has been proposed to function as an auxin receptor (Scherer, 2011), however, recent studies indicate that ABP1 might not be an essential component is auxin signalling (Gao et al., 2015)

Mechanistically in the absence of auxin, AUXIN RESPONSE FACTORS (ARFs) are under the negative regulation of AUX-IAA proteins. The presence of auxin causes the association of AUX-IAA and auxin F-box proteins, leading to the degradation of AUX-IAA through ubiquitin-mediated proteosomal degradation (Robert-Seilaniantz et al., 2011).

There appears to mostly be an antagonistic interaction between auxin and SA pathways, with auxin signalling playing an antagonistic role in SA mediated disease resistance and SA being

able to stabilise AUX-IAA (Fu and Wang, 2011; Robert-Seilaniantz et al., 2011; Wang et al., 2007). Auxin also appears to suppress JA signalling (Robert-Seilaniantz et al., 2011)

1.4.3 Gibberellin

Gibberellins (GAs) are diterpenoid acids and probably function in the same cells as in which they are produced (Ludwig-Müller, 2011). Similarly to auxins they have historically mostly been studied for their role in growth and development, with their role in plant defence beginning to emerge more recently (De Bruyne et al., 2014).

In Arabidopsis and rice, GA binds to GA INSENSITIVE DWARF 1 (GID1) causing further interaction with a class of transcriptional repressors known as DELLA proteins, typically considered to be growth repressing regulators. This complex in turn interacts with the GID2 SCF complex, resulting in ubiquitin-mediated proteosomal degradation of the DELLAs (De Bruyne et al., 2014; Robert-Seilaniantz et al., 2011).

In Arabidopsis, DELLA signalling could possibly act in plant-pathogen interactions by promoting JA signalling and supressing SA signalling (Navarro et al., 2008), generally resulting in GA promoting resistance against biotrophs and supressing resistance against necrotrophs. However, GA appear to play different roles in different systems, with GA signalling having the opposite role in pathogen resistance in rice (De Bruyne et al., 2014). It has been suggested that the effect on JA signalling could possibly be due to DELLAs being able to competitively bind to JAZ proteins and thus releasing MYC2 (Navarro et al., 2008)

1.4.4 Abscisic acid

Abscisic acid (ABA) is a terpenoid plant hormone, sensing and signalling of which involves multiple receptors and signalling pathways. It is heavily involved in a wide array of stress responses, as well as normal physiological processes (Robert-Seilaniantz et al., 2011; Wang and Irving, 2011).

A core ABA signalling pathway involves PYRABACTIN RESISTANCE (PYR) / REGULATORY COMPONENT OF ABA RECEPTOR (RCAR) family of the ABA receptors known as PYRABACTIN RESISTANCE LIKE (PYLs) (Robert-Seilaniantz et al., 2011). These receptors are located in the cytoplasm as inactive dimers. ABA binding causes dissociation into monomers and binding to PROTEIN PHOSPHATASE 2C (PP2C), resulting in inactivation of its negative regulation of SNF1-RELATED PROTEIN KINASE 2 (SnRK2). SnRK2 in turn can activate various downstream signalling, including ion channels and transcription factors (Wang and Irving, 2011). Another ABA receptor is the chloroplast located ABA-BINDING PROTEIN (ABAR) (Robert-Seilaniantz et al., 2011). Binding of ABA results in interaction with negative regulators of the WRKY transcription factor family. Further, ABA signalling can be regulated by proteosomal degradation via

ABA-induced degradation of KEEP ON GOING (KEG), a RING-finger ubiquitin E3 ligase targeting ABA INSENSITIVE 5 (ABI5). In Arabidopsis, ABI5 is a transcription factor involved in ABA signalling.

Due to its multifaceted role in responses to various environmental factors ABA has been proposed to play a key role in modulating the cross talk between biotic and abiotic stress (Lee and Luan, 2012). Often ABA has been seen to strengthen abiotic responses and weaken biotic responses (Robert-Seilaniantz et al., 2007). However, ABA appears to play different roles depending on infection phase and pathogen lifestyle (Ton et al., 2009). Early in defence, ABA can function by promoting physical defence barriers, for example, via ABA-dependent stomatal closure and possibly callose deposition.

Most likely ABA interacts mutually antagonistic with SA (Cao et al., 2011; Derksen et al., 2013; Robert-Seilaniantz et al., 2011), as well as inhibits BR signalling (Zhang et al., 2009). The interaction with JA /ET signalling appears more complex (Ton et al., 2009). Possibly ABA exerts a positive effect via the MYC branch of the JA pathway and a negative via the ERF branch.

There is ample evidence of interactions between ABA and ROS. In an ABA-deficient *Solanum lycopersicum* mutant, peroxidase activity was increased and exogenous application of ABA down-regulated the apoplastic peroxidase activity to WT levels (Asselbergh et al., 2008). Similar treatment had no effect on peroxidase activity in wild-type plants. Also, exogenous application of ABA to *A. thaliana* leads to induced accumulation of antioxidants, such as alpha-tocopherol and L-ascorbic acid (Ghassemian et al., 2008). Both ABA and ET stabilise DELLA proteins (Achard et al., 2006, 2007), this may in turn induce the expression of enzymes capable of detoxifying ROS (Grant and Jones, 2009). Interestingly, ABA-induced ROS generation in guard cells has been found to be an important component of guard cell closing (Mittler and Blumwald, 2015).

1.4.5 Cytokinins

Cytokinins (CKs) in plants are of the adenine-type and are typically involved in processes such cell division, organ differentiation and leaf senescence (Robert-Seilaniantz et al., 2011; Wang and Irving, 2011).

CKs are perceived by membrane-located histidine kinase proteins, in Arabidopsis by ARABIDOPSIS HISTIDINE KINASE 2 (AHK2), AHK3, and CYTOKININ RESPONSE 1/AHK4 (Lomin et al., 2012; Müller and Sheen, 2007; Wulfetange et al., 2011). Interaction with CKs leads to the receptors being autophosphorylated. The phosphorylation is transferred to cytosolic Arabidopsis histidine phosphotransfer proteins (AHPs), leading to their relocalisation to the nucleus where they phosphorylate and activate Arabidopsis response regulators (ARRs). ARRs can be both negative and positive regulators of CK signalling (Robert-Seilaniantz et al., 2011).

The role of CKs in plant immunity is complex and seems to mainly involve interaction with other phytohormonal pathways (Naseem et al., 2014, 2015b; O'Brien and Benková, 2013). Whereas SA inhibits CK mediated growth responses, there seems to be a synergism between CKs and SA in defence against biotrophs, with CKs increasing SA biosynthesis and signalling (Choi et al., 2010). JA signalling appears to suppress CK responses, but CKs appear to promote JA defence responses. There also appears to be a mutually antagonistic effect of auxin and CKs in modulating defence responses (Naseem et al., 2015b)

1.4.6 Brassinosteroids

BRs constitute a large class of polyhydroxysteroids that regulate cell expansion/division and cell differentiation (Wang and Irving, 2011). To achieve this, BRs seem to interact with other growth regulators such as GA and auxins (Fridman and Savaldi-Goldstein, 2013).

BRs are typically recognised by leucine-rich repeat receptor-like kinases located in the plasma membrane. In Arabidopsis, the main receptor for brassinolides (BLs) has been identified as BRI1 (Kinoshita et al., 2005; Robert-Seilaniantz et al., 2011). Binding of BL results in autophosphorylation and homo-dimerization of BRI1 and recruitment of BAK1. The signal is transduced via phosphor relay to the nucleus, where several transcription factors are affected (Clouse, 2011).

There is conflicting evidence as to the effect of BRs on plant defence, with BR signalling appearing to play a key role in balancing growth and development versus plant defence. In contrast to SA and JA/ET signalling, the effect of BRs seem to be mostly independent of plant species and pathogen lifestyle (De Bruyne et al., 2014). Exogenous application of BR appears to antagonize FLS2-mediated immunity (Albrecht et al., 2012; Belkhadir et al., 2012). The mechanism behind this antagonism is unknown, but appears to be downstream of BAK1 and possibly also by a separate pathway acting independently of BAK1. Further, there appears to be a narrow concentration range where BR can actually prime innate immunity rather than antagonise it (Belkhadir et al., 2012).

BRs seemingly interact with a wide range of hormonal pathways upon pathogen recognition, i.e. SA, JA, ET, ABA, auxins, and GA (De Bruyne et al., 2014). There is evidence of synergistic crosstalk between BRs and SA, as well as JA/ET and ABA (Divi et al., 2010). However, BRs negatively interact with JA in the regulation of growth processes and also seem to negate JA induced resistance to certain pathogens (Choudhary et al., 2012). It appears that BRs can also have a negative effect on SA signalling, as well as appear to suppress GA biosynthesis and activate GA repressor genes. (De Vleesschauwer et al., 2012).

BRs also affect the ROS homeostasis, with BR induced ROS production playing a central role in stress tolerance. As with many of its interactions with hormonal pathways, however, the effect of BRs on ROS can also be the opposite, by inducing antioxidant and ROS scavenging genes. (De Bruyne et al., 2014).

1.5 Reactive oxygen species

Photorespiration and various metabolic processes result in the generation of intracellular ROS typically in the form of hydrogen peroxides, hydroxyl radicals, reactive nitric species and superoxide radicals (Grene, 2002; Pitzschke et al., 2006). Plants are able to utilise ROS as signalling molecules in the regulation of numerous processes involved in development, adaptation to physiological conditions, as well as response to various stresses. The recognition of pathogens typically trigger a ROS burst that mediate defence signalling associated with such processes as the HR, SAR, cell wall protein cross-linking, defence gene activation and synthesis of phytoalexins (Bradley et al., 1992; Durrant and Dong, 2004; Lamb and Dixon, 1997; Levine et al., 1994; Torres, 2010).

Plants are able to control ROS production using various enzymes, the most well-known of these are the plasma membrane-localised NADPH oxidases RBOHs and the cell wall-localised apoplastic peroxidases (class III peroxidases, CIII Prxs) (Bolwell et al., 1999; Torres et al., 2002), both generating apoplastic ROS. However, ROS from metabolic origins and regulation of scavenging systems also participate in plant responses to pathogens (Mittler et al., 2004). Superoxide dismutases, catalases and ascorbate peroxidases, as well as antioxidants such as; ascorbate, glutathione and tocopherol play an important role in regulation of ROS levels (Foyer and Noctor, 2005).

RBOHs are transmembrane proteins capable of generating superoxide radicals via the oxidation of cytoplasmic NADPH (Welinder et al., 2002). In *A. thaliana*, two members of the RBOH family, RBOHD and RBOHF, are two of the main sources of ROS in response to pathogens (Torres et al., 2002). However, *atrbohD/F* double mutants are still able to produce ROS in response to wounding and pathogens.

CIII Prxs are part of a large family, for example there are 73 CIII Prxs in *A. thaliana* (Tognolli et al., 2002). CIII Prxs have both hydroxylic and peroxidative cycles and as such they are able to produce ROS as well as oxidise cell wall aromatic compounds (Francoz et al., 2015). They are involved in varied processes such as; lignification, plant growth and cell elongation, auxin metabolism, seed germination and defence against pathogens (Almagro et al., 2009; Hiraga et al., 2001; Shigeto and Tsutsumi, 2016). Altering the expression of these enzymes results in a varied effect on plant defence against pathogens (Shigeto and Tsutsumi, 2016). This is proposed to be due to CIII Prxs being able to both produce and consume ROS, depending on the reaction conditions and availability of substrates. Both proteomic and transcriptomic approaches have shown that CIII Prxs exhibit highly specific expression profiles and the importance of strict regulation of expression, both temporarily and spatially, has been seen (Francoz et al., 2015).

Like RBOHs, CIII Prxs are one of the major components in ROS production as part of the defence responses to pathogens (Soylu et al., 2005). Various CIII Prxs have been shown to be induced by pathogens, as well as exogenous application of JA, ET and SA, leading to an increase in peroxidase activity (Almagro et al., 2009; Lehtonen et al., 2009). CIII Prxs have

been shown to play a central role in plant defences, against both necrotrophs and biotrophs (Almagro et al., 2009).

2 MATERIALS AND METHODS

Materials and methods utilised in this study are presented in full in respective publications (I-II).

Method	**Publication**
Bacterial virulence and growth *in planta* assay	I, II
Callose staining	II
Cell wall fortification assay	II
Cloning, vector constructs, transformation	II
Comparative transcriptomics	I
Cuticular permeability assay	II
DNA/RNA extraction and purification	I, II
Gene clustering and enrichment analysis	I
Genome browsing using BLAST	I, II
Immunoblotting	I
Mutant screen	I, II
PCR	I, II
Peroxidase activity assay	II
Plant growth retardation assay	I, II
Quantitative ROS production analysis	I
Quantitative RT-PCR	I, II
RNA sequencing data analysis	I, II
ROS staining assays	II
RT-PCR	II
Statistical analysis	I, II

Organism	**Publication**
Arabidopsis thaliana	I, II
Botrytis cinerea Pers.: Fr strain B.05.10	II
Pectobacterium carotovorum ssp. Carotovorum SCC1	I, II
Pseudomonas syringae pv. *Tomato* DC3000	II

3 AIMS OF THE PRESENT STUDY

The aim of this study was to further elucidate the molecular mechanisms involved in DAMP signalling in *A. thaliana* using primarily short OGs released during the infection by the soft-rot pathogen *P. carotovorum* as the model DAMPs. The foundation of this work was two genetic screens for OG-insensitive mutants and transcriptomics in the form of RNA sequencing of plants treated with short OGs.

The following topics were explored:

- Comparative transcriptomic and phenotypic analyses of short OG signalling.
- Screening of T-DNA activation tagged mutants for altered OG responses.
- Characterisation of genes found to be involved in OG responses, in particular the CIII Prx PEROXIDASE 57 (PER57).

4 RESULTS AND DISCUSSION

4.1 Short oligogalacturonides play a role in plant innate immunity (I)

Resistance to broad host-range necrotrophs, such as Pectobacteria, appears to depend on the general plant innate immunity responses, such as SA- and JA/ET-mediated defences, triggered by recognition of MAMPs or DAMPs (Collmer et al., 2009; Norman-Setterblad et al., 2000; Toth and Birch, 2005; Toth et al., 2006). As one of the primary end products of PCWDEs action upon pectin, OGs have been extensively studied for their role as DAMPs (Bishop et al., 1981; Davidsson et al., 2013; Hahn et al., 1981). Several transcriptome analyses have studied long OGs with a DP above 10 (Denoux et al., 2008; Ferrari et al., 2007; Moscatiello et al., 2006). These studies indicate that long OGs are the most efficient at triggering plant defence responses, with shorter OGs being labelled as inactive. However, several early studies indicate that also short OGs could be potential activators of DAMP responses (Davidsson et al., 2013). Hence, we decided to investigate plant responses to short OGs, both on a transcriptomic level using RNA sequencing, as well as on a phenotypic level. As a model for short OGs we chose to use trimeric OGs (trimers), as these have been shown to be present at elevated concentrations during infections by necrotrophic pathogens and have a similar effect on particular responses in Arabidopsis when applied exogenously, as does polygalacturonic acid degraded with pectolytic enzymes, as well as culture filtrate from *P. carotovorum* (An et al., 2005; Montesano et al., 2001; Norman et al., 1999; Pontiggia et al., 2015).

4.1.1 Short oligogalacturonides effect the Arabidopsis transcriptome

At 3 hours post treatment the transcriptome exhibited significant differences between plants treated with trimers and mock treated plants. The RNA sequencing revealed 517 significantly up-regulated genes and 183 significantly down-regulated genes, compared to the mock treatment.

To further elucidate the type of genes involved in the response we performed a gene set enrichment analysis (GSEA). The GSEA suggested a trend of trimer-mediated up-regulation of biotic defence-related gene sets and a downregulation of gene sets related to growth and development. This is typical of what would be expected of a MAMP/DAMP-response, with defence responses being up-regulated at the cost of growth and development (Bolton, 2009; Gómez-Gómez et al., 1999).

To investigate the differences in response between short and long OGs we compared our data with published transcriptomic data from studies with a similar experimental set-up, but using long OGs and DNA microarrays (Denoux et al., 2008; Ferrari et al., 2007).

Interestingly, a significant overlap could be seen. However, the response to long OGs included a much larger set of genes, as well as a typically higher induction of similar genes. Further, a GSEA revealed that similar types of gene sets were affected. For both trimers and long OGs the expression of genes associated with the defence-related JA, ET and SA signalling pathways were typically enhanced, while the expression of genes involved in the GA and CK pathways, associated with development and growth, were mainly down-regulated. Notably, among the gene sets found to be specifically up-regulated by long OGs were respiratory burst (GO:0045730) and SAR (GO:0009627). This could be indicative of a difference in the response between long and short OGs.

It is essential to point out that the studies looking at long OGs used an OG treatment enriched in long OGs, but containing fractions of DPs ranging from 2-19 (unpublished data). Therefore, the difference could potentially be due to the triggering of several signalling pathways by the long OG mix.

4.1.2 Short oligogalacturonides do not trigger a ROS burst in Arabidopsis seedlings

Activation of plant defences by various elicitors, including long OGs, has previously been shown to be accompanied by a ROS burst caused primarily by the plasma membrane NADPH oxidase RBOHD (Galletti et al., 2008; Lamb and Dixon, 1997; Legendre et al., 1993). As indicated by the transcriptomic analysis, there could be a difference in the capacity to trigger the initial ROS burst by trimers and long OGs. A more detailed analysis of RBOHD expression using qPCR indicated that, even though both types of OGs induce expression, long OGs triggered a stronger and more prolonged expression.

In accordance with previous studies (Bellincampi et al., 2000; Legendre et al., 1993), our results confirm that short OGs are unable to trigger the RBOHD mediated ROS burst. The ROS burst is typically connected with HR-associated cell death (Lamb and Dixon, 1997; Torres, 2010). However, the RBOH-derived ROS have been shown to be capable of suppressing cell death in some situations (Torres et al., 2005). As the HR-associated cell death is considered to promote susceptibility to necrotrophs (Mengiste, 2012), it is thought-provoking to speculate that during the early stages of infection, when mostly long OGs have had time to form, the ROS burst would have a positive role in resistance. Whereas at later stages, when there are more PCWDEs present resulting in larger amounts of shorter OGs, the ROS burst and subsequent cell death could potentially have a negative effect on resistance.

It has been established that the RBOHD dependent ROS burst is not required for long OG induced resistance to *B. cinerea*, nor for expression of several OG-responsive genes (Galletti et al., 2008). Also, our comparative meta-data analysis show that long and short OGs influence the expression of a somewhat different set of peroxidases, which could possibly have an effect on ROS homeostasis. Therefore, it is not clear what affect the lack of the initial ROS burst, after trimer treatment, has on plant-pathogen interactions.

4.1.3 Short oligogalacturonides elicit plant defence responses

Overall, gene expression data indicates that pre-treatment with trimers could potentially have a protective role against pathogens. Contrarily, the inability of trimers to elicit a ROS burst, as well as the indication from the transcriptomic analysis that trimers do not upregulate gene sets related to SAR, might indicate that trimers are unable to prime plant defences against pathogen invasion. To test the *in planta* effect of exogenous application of trimers on defence we pre-treated plants with mock, trimers, or a mixture of long OGs, 24 hours before infection with *P.* carotovorum. Both types of OGs were able to induce plant defences and inhibit growth of the necrotrophic pathogen. Somewhat surprisingly trimers were able to do so to the same extent as the long OGs. One speculation could be that the relatively high inoculums of bacteria used in this experiment partially bypasses the early stages of infection where long OGs might be more efficient.

Recently it was shown that engineered *in vivo* release of OGs of varying DP lead to enhanced resistance to *P. carotovorum* in Arabidopsis (Benedetti et al., 2015). However, pre-treatment of Arabidopsis with long OGs did not lead to enhanced resistance against the same pathogen (Gramegna et al., 2016), conceivably indicating that shorter OGs play a larger role in resistance against *P. carotovorum*. Long OG signalling appears to go through the KAPP/GRP3/WAK1 complex and short OGs are possibly sensed differently. Recognition of OGs by WAK1 seems to require longer OGs and unlike the case for *B. cinerea* loss of GRP-3 or WAK1 overexpression does not affect resistance against *P. carotovorum*. Further, loss of KAPP leads to increased sensitivity to *P. carotovorum*, rather than the increased resistance observed against *B. cinerea*. It is tempting to speculate that shorter OGs play a larger role in resistance against bacterial necrotrophs, whereas longer OGs play a more significant role against necrotrophic fungi. However, such speculations require further research.

The fact that we were able to observe a priming effect against *P. carotovorum* with long OGs in our experiments might be due to small differences in experimental set-up, for example, there might be differences in timing of the infections or the use of Silwet in the pre-treatment solutions could potentially influence how accessible the OGs are to receptors in the plants. Again, it should be noted that Gramegna et al. 2016 used an OG mixture enriched in long OGs but also containing fractions of shorter OGs, this might trigger separate and conflicting responses in the plants.

4.1.4 Oligogalacturonides inhibit growth in Arabidopsis seedlings

As the transcriptomic data indicated a downregulation of genes related to processes involved in growth and development after treatment with both short and long OGs, as well as previous data demonstrating that treatment with flg22 results in growth retardation in Arabidopsis seedlings (Gómez-Gómez et al., 1999), we decided to test if this was also the case for OG treatment. In agreement with the hypothesis that there is a trade-off between plant defence

and plant growth and development (Bolton, 2009; Scheres and van der Putten, 2017), our data showed that both types of OGs were capable of inducing growth retardation in Arabidopsis seedlings. Surprisingly the growth retardation caused by short OGs was somewhat larger than that of long OGs, indicating that the two types of OGs trigger somewhat different responses.

4.1.5 Short oligogalacturonides trigger MPK3 and MPK6 phosphorylation in Arabidopsis seedlings

MAPK activation is an essential step in the activation of many defence responses triggered by both DAMPs and MAMPs (Rasmussen et al., 2012). In Arabidopsis there are 20 MAPKs (Ichimura et al., 2002), of which at least four are implicated in pathogen responses; MPK3, MPK4, MPK6 and MPK11 (Bethke et al., 2014). The separate MAPK cascades (MPK3/MPK6 cascade and MPK4 cascade) have a different regulation depending on which PRR it is that recognises its respective DAMP/MAMP and triggers the cascade (Xu et al., 2014).

The phosphorylation of MPK3/MPK6 has previously been demonstrated in response to long OGs (Denoux et al., 2008; Mattei et al., 2016). It has also been demonstrated that OG- and flg22-induced defence against *B. cinerea* are dependent on these two MAPKs (Galletti et al., 2011).

It is known that exogenous application of hydrogen peroxide triggers MAPK activation (Kovtun et al., 2000). However, it was recently demonstrated that in flg22-signalling the RBOHD dependent ROS burst and MPK3/MPK6 activation can function as two independent signalling pathways (Xu et al., 2014). Therefore, we decided to test if short OGs were capable of triggering MAPK activation. Indeed, similarly to long OGs, exogenous application of timers triggered MPK3/MPK6 phosphorylation in Arabidopsis seedlings. At lower concentrations, the degree of phosphorylation appears to be somewhat less for trimers, indicating a potentially weaker response. Long OGs caused similar levels of phosphorylation independently of concentration, implying saturation of the response already at lower concentrations. At higher concentrations, the phosphorylation appears to be similar for both short and long OGs, with neither of them causing as high degree of phosphorylation as flg22.

4.2 Peroxidase-generated apoplastic ROS impair cuticle integrity and contribute to DAMP-elicited defences (II)

4.2.1 Screening for mutants with altered OG-sensitivity

The current view is that there exists a trade-off between plant defence and plant growth and development (Bolton, 2009). Recognition of a pathogen, or activation of the plant immune system in any other manner, would according to this view lead to the plant prioritising defence at the expense of growth. The inability to recognise an assaulting pathogen would lead to higher growth rates, but higher sensitivity to the invading pathogen and consequently potentially higher losses. This pathogen-induced growth retardation is known to be triggered when treating Arabidopsis seedlings with the MAMP flg22 peptide (Gómez-Gómez et al., 1999). We established that also various DPs of the DAMP OG had a similar retarding effect on Arabidopsis seedling growth and utilised this fact to develop two forward genetic screens to identify and characterise OG-responsive components in DAMP signalling (Figure 2).

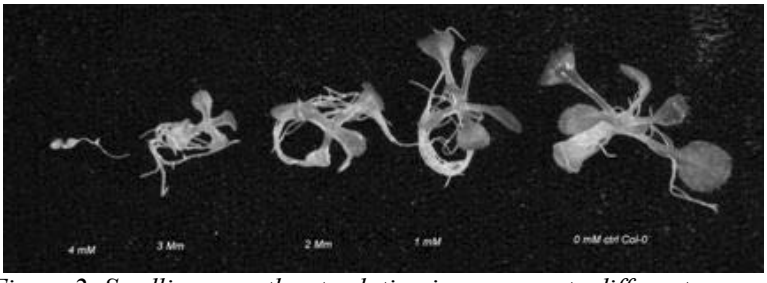

Figure 2: Seedling growth retardation in response to different concentrations of OGs (DP2-19.

Two ecotypes of *A. thaliana* (Col-0 and C-24) were used to establish two separate libraries of T-DNA activation lines using the pSKI015 vector (Weigel et al., 2000), containing four Cauliflower mosaic virus 35S enhancers. These were in turn used to set up two forward genetics screens (Figure 3). In both screens a mixture of OGs with a varied degree of polymerisation (DP 2-19) was used. An endopolygalacturonase (PehA) was used to degrade commercially available polygalacturonic acid (Saarilahti et al., 1990). The concentration of the resulting OG-mixture was estimated by comparison with commercially available trimers on aluminium Silica gel 60_{F254} TLC-plates and analysed using mass spectrometry. The first screen looked for highly tolerant seedlings after infiltration with the OG mixture. In the second screen, seedlings were grown individually in liquid ½ MS on 96-well plates, allowing for a detailed observation of OG-triggered growth retardation. All mutants with altered growth response were further screened for developmental phenotypes and altered sensitivity/resistance to *B. cinerea*, *P. carotovorum* and *P. syringae* (Table 1).

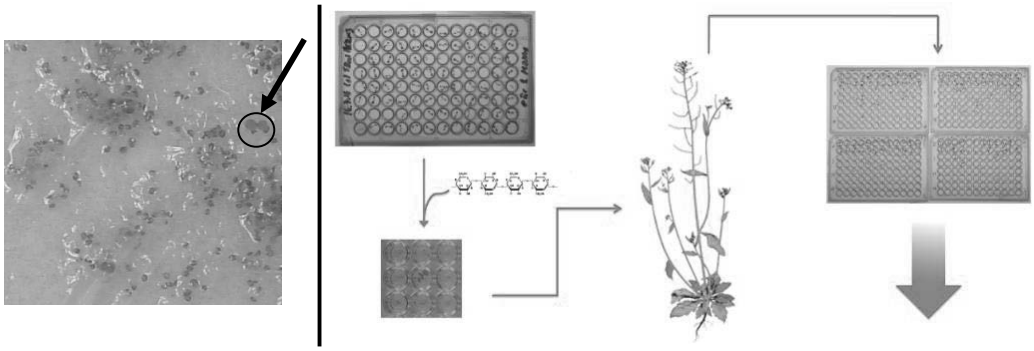

Figure 3: Left: First screen. Seedlings after infiltration with OGs. The circle indicates an example of a tolerant seedling. Right: Second screen. Plants were grown on 96-well plates and then treated with OGs. Seedlings exhibiting resistance or sensitivity were selected, grown for seeds and subjected to a second more detailed round of screening.

Genotype	Screened T-DNA lines	Candidate mutant lines	Identified insertions	Insertion in gene	Pathogen phenotype	Developmental phenotype
Col-0	35000	30	19	7	10	3
C-24	62400	46	22	12	18	3
Total	**97400**	**76**	**41**	**19**	**28**	**6**

Table 1: Overview of the outcome from two forward genetics screens looking for altered OG induced growth retardation in A. thaliana seedlings.

One candidate mutant line from the second screen exhibiting hypersensitivity to OGs (OG hypersensitive – *ohy1*), resistance to the necrotrophic pathogens *B. cinerea* and *P. carotovorum*, as well as sensitivity to the hemibiotrophic pathogen *P. syringae*, was selected for further studies.

Utilising co-segregation analysis, qPCR and next-generation sequencing we established that the phenotype was due to an insertion causing overexpression of a CIII Prx – PEROXIDASE 57 (PER57). We further verified that overexpression of PER57 was behind the observed phenotypes by overexpressing PER57 in wild-type plants.

In line with *ohy1* overexpressing a peroxidase we observed heightened levels of hydrogen peroxide (DAB staining) and super oxide (NBT staining) in untreated leaves. To test if RBOHD contributed to the heightened ROS levels we treated the plants with diphenylene iodonium (DPI), a known inhibitor of the NADPH oxidase-dependent oxidative burst. The treatment resulted in no noticeable effect on ROS formation, indicating peroxidases as the primary source of these ROS.

4.2.2 Overexpression of per57 increases the cuticle permeability and primes OG related responses

As the observed phenotypes implied that overexpression of PER57 might be effecting the leaf cuticle (L'Haridon et al., 2011), we tested our mutants with toloudine blue (TB) and calcofluor white staining. Our results indicate that the PER57 overexpression leads to increased cuticle permeability. This was further shown to lead to decreased cell wall fortification against PCWDEs, as assayed by putting drops of *P. carotovorum* culture filtrate on leaves and observing the resulting maceration.

We further verified that genes related to cutin formation and cutin biosynthesis were strongly down-regulated in the PER57 overexpressing plants. In line with results from other mutants with impaired cuticle integrity (Voisin et al., 2009), the expression of cuticular wax biosynthesis genes were up-regulated.

Looking at defence marker genes we were able to establish that PER57 overexpression led to priming of OG related response genes, but not of several SA and JA related response genes. This was true for both OG-induced gene expression as well as flg22-induced gene expression. To see if this effect was simply a direct effect of the perturbed cuticle we infiltrated leaves with either elicitor and measured the resulting callose accumulation. Increased callose accumulation was observed for both elicitors compared to wild-type, indicating that the primed responses are not due to the elicitors being able to diffuse easier across the more permeable cuticle. As OG signalling is a key component in resistance to necrotrophs (Davidsson et al., 2013), these results could explain why plants exhibiting cuticular perturbations are more resistant to necrotrophs. The sensitivity to the (hemi)biotrophic pathogen *P. syringae* could possibly be due to decreased physical barriers or interference with defence signalling. This still remains to be elucidated.

4.2.3 PER57 as a model of CIII Peroxidases in plant defence

As CIII Prxs exist as a large family, with partially redundant functions, where the functions are to a large extent dependent on spatiotemporal regulation (Cosio and Dunand, 2009; Shigeto and Tsutsumi, 2016; Tognolli et al., 2002), and we had found multiple peroxidases to be up-regulated in response to OGs, we wanted to test if the phenotypes resulting from overexpression of PER57 could be a general effect of overexpressing peroxidases. Transgenic lines overexpressing six different apoplastic CIII Prxs, three (PER10, PER28, and PER34) that we had found to be responsive to OGs and three (PER44, PER53, and PER64) that we had found to be non-responsive to OGs were generated. Overexpression of all six CIII Prxs generated phenotypes identical with that of PER57 overexpression, including; growth phenotype, increased levels of superoxide, increased cuticle permeability and resistance to *B. cinerea*. Similar results have been observed when overexpressing PER71 (Chassot et al., 2007; Raggi et al., 2015). As such, our results indicate that these responses are general for overexpression of CIII Prxs and that PER57 could function as a general model for studying the involvement of CIII Prxs in plant responses to pathogens. Interestingly

neither *per57*, nor *per71* mutants exhibit a pathogen phenotype. This indicates an overlapping role of CIII Prxs. The *per33*/*per34* knockdown plants, however, exhibit reduced defence responses in response to MAMPs and subsequent enhanced susceptibility to a broad range of fungal and bacterial pathogens (Daudi et al., 2012; O'Brien et al., 2012).

4.2.4 Peroxidases and ABA

Due to the influences of ABA on cuticle formation (Asselbergh et al., 2007; L'Haridon et al., 2011), we sought to investigate any possible involvement of ABA in the responses mediated by CIII Prxs. We found that ABA treatment of peroxidase overexpressing lines restored genes related to cutin formation and cutin biosynthesis to the same levels as wild-type, whereas genes related to cuticular wax biosynthesis were even more up-regulated than in the mock treated overexpression lines. Furthermore, ABA treatment was able to completely abolish the ROS accumulation and restore the leaf cuticle in peroxidase overexpressing lines. This was accompanied with wild-type level susceptibility to *B. cinerea*.

In agreement with previous research (Gonzalez-Guzman et al., 2012; L'Haridon et al., 2011), the ABA- deficient *aba2* and ABA-insensitive *pyr/pyl 112458* sextuple mutant exhibited similar phenotypes to CIII Prx overexpression lines in regards to ROS formation, cuticle permeability and resistance to *B. cinerea*. As expected, ABA treatment was only able to restore the wild-type phenotype for the *aba2* mutant. Under normal growth conditions both ABA mutants and peroxidase overexpressors exhibited higher peroxidase activity than observed in the wild-type. ABA treatment reduced the peroxidase activity in all mutants except for the *pyr/pyl 112458* mutant, but it was only able to completely restore activity to wild-type level in the *aba2* mutant. Noticeably, the peroxidase activity remained high in the peroxidase overexpression lines, indicating that while exogenous application of ABA was able to remove the ROS produced by the peroxidases it only had a minor direct effect on the activity of the peroxidases.

Our results, combined with previous research on cuticular and ABA mutants lead us to propose that cuticle integrity is influenced by a positive feed-back loop, where a disturbed cuticle leads to elevated ROS levels via increased peroxidase activity, which in turn leads to impaired cuticle formation and biosynthesis. Under normal circumstances this loop is regulated by ABA. In the situation where a necrotrophic pathogen is invading the plant, recognition of cell-wall derived DAMPs, such as OGs, leads to activation of peroxidases that further promote resistance signalling via the creation of ROS. Some necrotrophs, such as *B. cinerea*, are capable of inducing ABA production in plants, as well as producing ABA and antioxidants such as oxalic acid themselves, possibly to facilitate invasion via removal of the ROS generated by peroxidases (Kettner and Dörffling, 1995; L'Haridon et al., 2011; Siewers et al., 2006).

5 CONCLUSIONS AND FUTURE PROSPECTS

This work has demonstrated that the neglected shorter OGs are able to function as biologically active DAMPs. This was seen by their ability to induce qualitatively similar defence-related gene expression and lower growth-related gene expression as longer OGs. We showed that, analogously to MAMPs, short and long OGs are capable of inhibiting seedling growth. Priming with short and long OGs lead to induced resistance in Arabidopsis against *P. carotovorum*. We also demonstrated that the defence signalling elicited by short OGs might in part go via MAPKs, similarly to long OGs, but not via the general ROS burst that is triggered by long OGs. This shows that even though there are many similarities between short and long OG signalling there are distinct differences.

Intriguingly, the generally larger effect of the long OGs on the transcriptome, trigger of ROS burst, and possibly stronger MAPK activation, was not reflected in our phenotypical experiments on growth retardation and priming of defences against *P. carotovorum*.

How short OGs are sensed remains elusive, as sensing does not appear to go via WAK1 (Gramegna et al., 2016). Several uncharacterised receptors and membrane-associated proteins were found in the transcriptomic data, as well as among the generated mutants with altered OG sensitivity. These are potentially interesting candidates as the missing receptor(s) for short OGs, as well as possible additional receptors for long OGs.

Our gene expression data shows that several CIII Prxs are up-regulated in response to OGs. We found that overexpressing CIII Prxs led to reduced expression of genes known to be involved in cuticle biosynthesis and subsequent increased cuticle permeability, increased ROS production and resistance to necrotrophic pathogens. Previously the cuticular mutants *bdg* and *lacs2.3* have been shown to exhibit similar phenotypes (Bessire et al., 2007; Jakobson et al., 2016; L'Haridon et al., 2011; Schnurr et al., 2004). We propose a feedback mechanism influencing cuticle integrity. Impaired expression of cuticle related genes leads to heightened ROS production and increased ROS production via CIII Prxs lead to further decreased gene expression. ABA appears to function as a major regulator in this feedback loop. Mutants deficient in ABA signalling or synthesis exhibit similar phenotypes as cuticular mutants and CIII Prx overexpression lines. ABA treatment of these mutants and overexpression lines alleviate the observed phenotypes.

As previously reported, the OG signalling pathway plays a significant role in resistance to necrotrophic pathogens (Davidsson et al., 2013; Ferrari et al., 2013; Ridley et al., 2001). The enhanced resistance to *B. cinerea* observed in plants with cuticular defects was found to be independent of SA, ET and JA signalling (Chassot et al., 2007). Based on our findings we propose that such signalling at least in part goes through the OG signalling pathway. Overall, our research highlights the importance of peroxidases in both DAMP and MAMP responses. Increased peroxidase activity leads to primed expression of genes associated with the OG-

dependent defences, which are at least partially independent of the SA and JA signalling pathways, resulting in increased resistance to necrotrophic pathogens.

Interestingly, signalling by short oligogalacturonides appear to share some similarities with signalling triggered by short cellulose oligomers (Souza et al., 2017). Both types of DAMPs, composed of short cellulose oligomers, trigger MAPK phosphorylation and primes plant defences, whilst not triggering the characteristic ROS burst. If these similarities extend to other responses would be interesting to investigate and could lead to a deeper understanding of the plant innate immune system.

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Helsinki

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